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Plant Growth Promoting Rhizospheric *Bacillus* spp. Isolated from Black Pepper (*Piper nigrum* L.)

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ABSTRACT

Background: The rhizosphere contains many beneficial microorganisms which have a positive action on plant growth and development. In both natural and controlled ecosystems, beneficial plant-associated bacteria play a key role in supporting and increasing plant health and growth.

Methods: In this study, plant growth promoting *Bacillus* isolates associated with the rhizosphere of black pepper (*Piper nigrum*) were selected and used for bioassay experiments for the host plant as well as for two test plants *viz*. French bean (*Phaseolus vulgaris*) and Pea plant (*Pisum sativum*).

Result: This investigation revealed that the isolates chosen for the study exert a positive effect on the growth of inoculated black pepper plants and the test plants as compared to uninoculated control. Molecular identification of the selected isolates was done based on 16S rRNA gene analysis. All the four isolates exerted a considerable influence on the growth of black pepper cuttings and there is a significant increase in the shoot height and root length compared to untreated cuttings. Seed bacterization of the two test plants resulted in the increment of plant height and a heavier fresh weight and dry weight when compared to control plants. Based on these findings, it is evident that the isolates can be employed to enhance the growth not only of black pepper but of the test plants as well. Hence, according to this study, the selected isolates could be used as plant growth-promoting inoculants to attain the desired results of stem cuttings and seed bacterization.

Key words: *Bacillus*, Black pepper, PGPR, *Phaseolus vulgaris*, *Piper nigrum*, *Pisum sativum*.

INTRODUCTION

Piper nigrum L. belonging to *Piperaceae* family, commonly known as black pepper is an important crop plant popularly used in both medicinal and culinary world. It has been termed as *black gold on vines* and rightly so, as the monetary benefits from it is immense and is the world's most traded spice as it is one of the most common spice added to cuisines around the world. Black pepper is a spicy, aromatic plant with carminative and antioxidant properties, known for its antiperiodic, anti-inflammatory and anti-cancer effects (Meghwal and Goswami, 2012). It has been used as a spice in almost all cuisines the world over. It is favoured not only in India but also in West Asia, Arabic countries, Greece, North west Africa, *etc*. (Scott *et al*., 2008).

Knowledge of the native microbial population, their characterization and identification is required for understanding the distribution and diversity of indigenous bacteria in the rhizosphere of specific crops (Keating *et al*., 1995). With increasing awareness about the chemical-fertilizers-based agricultural practices, it is important to search for region-specific microbial strains which can be used as growth promoting or growth enhancing inoculums to achieve desired crop production.

MATERIALS AND METHODS

Sample collection

The rhizospheric soil sample of *P. nigrum* was collected from Wahkdait Village under Pynursla Block, East Khasi Hills District of Meghalaya (25.2084°N, 91.9853°E). The soil attached on the roots was transferred into sterile plastic bags, they were marked and labelled and then brought to the Microbial Ecology Laboratory, North-Eastern Hill University, Shillong, Meghalaya, for processing.

Rhizobacteria were isolated by serial dilution method, 10 ml of sterile distilled water was taken in a tube and 1 g of rhizospheric soil was added to it. The tube was then shaken for 5 minutes and 10^{-6} grades dilutions were made, out of these, dilution grades of 10^{-4}, 10^{-5} and 10^{-6} 100 ml was aliquot on plates containing nutrient agar and spread properly with the help of an L-shaped spreader. The inoculated plates were then incubated for 24-48 h at 30±2°C. Bacterial isolates grown were then purified, maintained and stored for further studies.

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In-vitro screening for PGP traits

Ammonia production

Using the technique described by Cappuccino and Sherman (1992), the qualitative and quantitative production of ammonia was determined. Bacterial isolates were added to test tubes with 5 ml of peptone water and the test tubes were then cultured at 30±2°C for 48 h. After 48 h, the culture was vortexed for 5 minutes at 10,000 rpm and 1 ml of Nessler's reagent was added to the supernatant, where ammonia positive supernatants show an appearance of a yellow to brownish colour. A standard curve of ammonium sulphate was made and the amount of ammonia produced was calculated using the standard curve after being measured spectrophotometrically at 450 nm with the Lambda 35 UV-VIS spectrophotometer (PerkinElmer, USA).

Indole acetic acid (IAA) production

The bacterial isolates were screened for IAA production using Salkowski's reagent-based spectrophotometric technique (Brick *et al*., 1991). Isolates were cultured for 72 h at 30°C in Luria-Bertani broth that had 100 g of tryptophan added to it. Each of the supernatants was combined in a 1:2 ratio with Salkowski's reagent (49 ml of 35% perchloric acid and 1 ml of 0.5 M $FeCl_3$ solution). By using a Lambda 35 UV-VIS spectrophotometer, the optical density at 530 nm was evaluated to determine whether IAA was being produced successfully. The amount of IAA produced was determined using the IAA standard curve (Himedia, India).

Phosphate solubilization

Pikovskaya's agar medium with 0.04% Bromocresol green as a pH indicator was used to determine phosphate solubilizing activity. The isolates were inoculated on the said media and incubated at 30±2°C. Isolates that can solubilize phosphate formed a clear halo zone around the inoculated spots. Pikovskaya broth containing 1000 g ml^{-1} tricalcium phosphate (Subbarao, 1988) was used to determine a quantitative assessment of solubilized P. Using a spectrophotometer, the absorbance was measured at 430 nm for all the isolates. Using potassium dihydrogen phosphate as a standard, the concentration of solubilized P of the isolates was calculated from the standard curve. The pH of the broth was also measured and recorded.

Siderophore production

Bacterial isolates were inoculated on the Chrome Azurol S (CAS) agar plates to detect siderophore production by Schwyn and Neilands (1987) method. After 48-72 h of incubation at 30±2°C, isolates that can produce siderophore changes the colour of the media to orange and this conspicuous halo zone encircled the inoculated spots. Siderophore concentration was quantified using the CAS-Shuttle assay (Payne, 1994), which involved mixing 0.5 ml of culture supernatant with 0.5 ml of CAS reagent and measuring the absorbance at 630 nm in comparison to a reference made up of 0.5 ml of uninoculated broth and 0.5 ml of CAS reagent and the following formula is used to quantify siderophore production:

$$\text{Siderophore (\%)} = \frac{A_{reference} - A_{sample}}{A_{reference}} \times 100$$

Where

$A_{reference}$ = Absorbance of the reference.

A_{sample} = Absorbance of the sample.

Cellulase activity

Following the technique reported by Cattelan *et al*. (1999), cellulase production was screened by inoculating the bacterial isolates on a 1% carboxymethylcellulose (CMC) supplemented nutrient agar plates. After 48-72 hours of incubation at 30±2°C, 0.1% congo red solution was added on the plates for 15 minutes, then 1 M NaCl solution was used to washed the stain.

Protease activity

The production of protease was screened following Smibert *et al*. (1994), where bacterial isolates were inoculated on plates containing skim milk agar. The inoculated plates were then incubated at 30±2°C for 48-72 h, protease positive isolates shows a halo zone around the colonies.

Molecular identification of selected PGPR

The DNA of the selected isolates was extracted using the lysozyme method for molecular identification. 2 ml overnight culture was taken in a tube and spin for 5 minutes at 13,000 rpm. Sterile distilled water was used to wash the pellet twice. 3.3 µl of 3 mg ml^{-1} lysozyme and 96.7µl of sterile water were added to the pellet and vortexed. The tube was incubated for 1 hour at 37°C and 5 minutes at 95°C. After 5 minutes of centrifugation at 13,000 rpm, the supernatant was transferred to a new tube. PCR amplification was performed for 16S rRNA gene using universal primers 27F 5′ AGAGTTTGATCMTGGCTCAG 3′ and 1492R 5′ TACGGYT ACCTTGTTACGACTT3′. A PCR mixture with containing 1X PCR buffer (1.5 mM $MgCl_2$), 200 µM dNTPs, 0.2 µM of forward and reverse primers, 3U Taq DNA polymerase and 50ng of template DNA making a final volume of 25 µl was performed in a thermal cycler (Bio-Rad C1000 touch, USA). The amplicons were separated on an ethidium bromide (10 mg ml^{-1}) stained agarose gel (1%) using 1X tris-borate-EDTA as a buffer. Sequencing was done on the amplified products by Eurofins Scientific, India and the nucleotide sequences obtained were then run in NCBI BLAST to identify the isolates.

Bioassays

The four isolates were selected to be used as PGPR inoculant. The bacterial isolates were grown in a nutrient broth (250 ml) and incubated at 30°C for 48-72 h in a shaker incubator (120 rpm). For uniformity of all the isolates, distilled water was used to obtain a 10^8 CFU ml^{-1}.

Black pepper bioassay

Stem cuttings (15 cm in height) of black pepper were obtained from runner shoots of healthy plants. For bioassays,

the disease-free cuttings were surface-sterilized with ethanol (70%) for 5 min followed by several washings with ample amounts of sterile water and excess water adhering to the cuttings was removed using sterile tissue paper. Fresh cuts were made at the ends of the cuttings to yield sterile end tissues. The lower half of the stem cuttings, including the first node, was dipped in water (control) or bacterial suspensions (10^8 CFU ml^{-1}) for 30 minutes prior to transplanting to the pots. The height of the new black pepper shoots was scored after 90 days of plant growth.

Test plants bioassay

Additionally, if the bacterial isolates have beneficial effects on the host plant, *Piper nigrum*, they can also be employed to determine whether they have comparable impacts on the growth of other plants. Hence, bioassay was also performed on two test plants *viz* French bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*). The starting material (seeds) of both the test plants were sterilized for 1 minute in 3% sodium hypochlorite solution and rinsed properly (5-7 times) with ample distilled water. Post sterilization, the seeds were bacterized by soaking in the inoculant overnight. The bacterized seeds were then planted in potting bags containing sterilized soil. Re-application of inoculants @10^8 CFU ml^{-1} was done at 30 days after planting (Kharshandi *et al*., 2021) and the growth parameters of the test plants were analysed at maturity.

Statistical analysis

This entire study was performed between the year 2020 to 2022 and the data obtained from this study was analysed using standard statistical methods *viz.* OriginPro 2021 and MS-Excel 2010 (Microsoft, Washington, DC, USA). ANOVA and Tukey test with a p-value ≤0.05 were used to evaluate the effects of PGPR on the growth of test plants and black pepper.

Results and Discussion

Isolation of rhizobacteria

In this study, 48 rhizospheric bacterial isolates were isolated from *P. nigrum* (Table 1).

In vitro evaluation of PGP traits

Screening of PGP traits

Plant growth promoting attributes of the isolated bacteria were examined and it resulted to 47 isolates with positive results for ammonia and IAA production, 19 and 36 isolates for phosphate solubilisation and siderophore production, respectively.

Quantitative estimation of PGP traits

The production of ammonia, IAA, solubilized phosphate and siderophore were further quantified by subjecting the positive isolates to the appropriate tests. The isolates produced 0.12-2.75 µmol ml^{-1} ammonia, 0.7-92.5 µg ml^{-1} IAA, 4.59-30.28 µg ml^{-1} solubilized phosphate and 12.23-42.2% siderophore (Table 1).

Among the four isolates used for bioassay, isolate N42 shows maximum IAA and siderophore production of 31.8±0.2 µg ml^{-1} and 40.75±0.14%, respectively; isolate N16 shows maximum value for ammonia production 1.45±0.01 µmol ml^{-1} and isolate N17 shows maximum phosphate solubilisation of 14.41±0.19 µg ml^{-1}.

Diverse microorganisms present in the soil including bacteria (Arshad and Frankenberger, 1991; Khalid *et al.*, 2004), filamentous fungi (Kaldorf and Ludwig-Muller, 2000; Le Floch *et al*., 2003) and yeasts (El-Tarabily, 2004) are capable of producing physiologically active quantities of auxins and these have positive effects on the growth and development of plant.

Nutrient uptake by the plant is maximised by the increased number, length and volume of roots and all this is made possible by the ability of PGPR to produce IAA (Ramos *et al*., 2008). Reetha *et al.* (2014) exhibited the positive effect of *Bacillus subtilis* and *Pseudomonas fluorescens* on inoculated seeds of onion. Siderophores, which are iron-chelating compounds, secreted by microorganisms under iron-stressed conditions, play an important role in the positive association between microorganisms and the rhizosphere. (Dertz *et al*., 2006). PGPR have been reported to increase the growth of root and shoot through ammonia production and biomass production through nitrogen accumulation. The ability to solubilize inorganic phosphate is one of the major characteristics of a PGPR. During solubilisation, the bound inorganic calcium phosphates are released and also the bacteria produce organic acids that lower the pH of the medium (Gaind and Gaur, 1989). This is evident in our study as there is a decrease in pH in all the four selected isolates as compared with the uninoculated control after incubation (Fig 2).

Production of hydrolytic enzymes

Production of hydrolytic enzymes by the isolates was examined, where there are 10 cellulase producing isolates and 15 protease producing isolates (Table 1). Hydrolytic enzymes produced by PGPR such as cellulase and protease may aid in the breakdown of organic materials and nutrient mineralization and facilitates ingress of microorganisms into host tissues.

Molecular identification

Amplification of the 16S rRNA gene aids in the molecular identification of the four designated isolates. Using NCBI BLAST analysis, the isolates were identified as *Bacilus thuringiensis* strain MRHKN16 (1121 bp), *B. cereus* strain N17 (1135 bp), *B. mycoides* strain N42 (1359 bp) and *B. subtilis* strain MRHKN43 (1047 bp) and their NCBI GenBank accession numbers are OP314161, OP314191, OP133280 and OP314519, respectively.

Effects of PGPR treatment on plant growth

The four selected *Bacillus* spp. (N16, N17, N42 and N43) were subjected to bioassay for the host plant as well as for the two test plants and in both cases, the treated plants

Table 1: PGP traits of the isolates.

Isolates	Ammonia production (µmol ml^{-1})	IAA production (µg ml^{-1})	Phosphate solubilisation (µg ml^{-1})	Siderophore production (%)	Cellulase activity	Protease activity
N1	1.91±0.01	3±0.11	8.37±0.19	19.2±0.1	-	-
N2	1.30±0.01	12.66±0.14	-	20.6±0.12	-	-
N3	1.13±0.01	4.34±0.2	-	-	-	-
N4	0.52±0.03	3.7±0.1	-	-	-	-
N5	-	1.24±0.3	7.18±0.16	19.87±0.12	-	+
N6	1.06±0.01	10.65±0.2	18.5±0.24	22.52±0.3	-	-
N7	1.40±0.01	10.5±0.06	28.11±0.2	21.7±0.16	-	+
N8	0.80±0.02	2.7±0.1	23.23±0.09	23.34±0.1	-	-
N9	1.56±0.01	64.04±0.4	-	-	-	-
N10	1.88±0.01	35±0.2	-	13.41±0.11	-	-
N11	2.53±0.01	92.5±0.1	-	32.3±0.2	+	+
N12	2.13±0.03	11.54±0.2	-	23.35±0.08	+	+
N13	1.87±0.01	53.05±0.1	-	13±0.4	+	+
N14	2.35±0.04	28.5±0.2	-	25.1±0.12	-	-
N15	1.14±0.01	10±0.2	-	37.61±0.16	+	+
N16	1.45±0.01	9±0.2	14.35±0.19	35.04±0.17	+	+
N17	0.86±0.01	3.7±0.1	14.41±0.19	32.8±0.18	+	+
N18	1.70±0.01	34.3±0.2	25.77±0.16	23.34±0.1	-	-
N19	2.08±0.03	65.04±0.2	8.98±0.14	21.2±0.13	-	-
N20	1.26±0.02	3.5±0.1	-	19.74±0.22	+	+
N21	1.44±0.04	2.03±0.2	-	-	-	-
N22	1.64±0.01	14.8±0.3	-	18. ±0.3	-	-
N23	1.38±0.03	26.1±0.2	-	-	-	-
N24	1.54±0.01	3.8±0.3	-	-	-	-
N25	1.30±0.03	8.5±0.3	-	15.5±0.12	-	-
N26	0.17±0.01	64±0.2	-	-	-	-
N27	2.12±0.01	14±0.2	-	12.23±0.2	-	-
N28	1.28±0.01	1.5±0.1	-	-	-	-
N29	2.00±0.03	9.2±0.3	30.28±0.08	40.27±0.2	+	+
N30	0.30±0.02	35.7±0.3	-	-	-	-
N31	1.70±0.01	6.85±0.2	-	-	-	-
N32	2.08±0.02	2.3±0.2	-	-	-	-
N33	1.51±0.02	26.7±0.2	18.5±0.14	25.5±0.17	-	-
N34	1.75±0.02	-	-	-	-	-
N35	1.68±0.01	1.45±0.4	-	21.4±0.12	-	-
N36	1.64±0.03	1.6±0.3	7.27±0.04	21.5±0.07	-	-
N37	2.20±0.04	27.2±0.2	5.61±0.06	16.5±0.06	-	-
N38	1.43±0.20	24.4±0.2	-	19.11±0.12	-	-
N39	2.75±0.02	42±0.3	5.08±0.15	25.82±0.15	-	-
N40	1.30±0.02	39.5±0.3	13.37±0.08	42.2±0.33	-	+
N41	1.94±0.03	11.7±0.2	15.16±0.06	15.15±0.04	-	-
N42	0.70±0.01	31.8±0.2	12.24±0.09	40.75±0.14	-	+
N43	0.65±0.01	9.7±0.2	8.25±0.15	28.95±0.38	+	+
N44	0.12±0.01	0.7±0.1	-	16.1±0.32	+	+
N50	0.40±0.03	1.1±0.3	-	22.03±0.15	-	-
N51	0.50±0.01	4.5±0.3	4.59±0.05	33.78±0.1	-	-
N52	0.70±0.02	1.05±0.2	-	33.57±0.15	-	-
N53	1.60±0.01	10.6±0.2	-	14.64±0.25	-	+

*Values are means of three replicates, ± SE; (+) indicate positive reaction; (-) indicates negative reaction.

exhibit desirable results in terms of plant growth as compared to untreated plants.

All the four isolates exerted a considerable influence on the growth of black pepper cuttings and there is a significant increase at $p \leq 0.05$ in the shoot height and root length when compared to untreated cuttings. The cuttings treated with the four selected *Bacillus* isolates show a significant increase in the total plant height compared to untreated cuttings (Fig 1). Cuttings treated with *B. cereus* N17 and *B. mycoides* N42 isolates show a significant increase in shoot height and significantly improves root length as well. The positive enhancement and improved vigour of black pepper cuttings by *Bacillus* spp. was reported by Dastager *et al.* (2011).

The four isolates were also used in seed bacterization of the two test plants which result in the increment of plant height and a heavier fresh weight and dry weight when compared to non-bacterized plants (control). For French bean bioassay, the height of the plant was significantly increased in all the individuals treated with the *Bacillus* isolates (*B. thuringiensis* MRHKN16, *B. cereus* N17, *B. mycoides* N42 and *B. subtilis* MRHKN43), whereas; *B. thuringiensis* MRHKN16, *B. mycoides* N42 and *B. subtilis* MRHKN43 treated seeds give rise to plants with a heavier fresh weight and a heavier dry weight compared to untreated control (Fig 3). Co-inoculation of PGPR together with *Rhizobium* improved the plant growth, physiological and quality attributes and seed production of French beans (Yadegari and Rahmani, 2010 and Yadav and Raverkar, 2021). Saxena *et al.*, (2013) illustrate the use of *Bacillus* spp. as bio-inoculant along with bio-char which resulted in significant improvement in overall plant growth of French bean. For pea plant bioassay, it was found that there is a significant increase in plant height on plants treated with *B. mycoides* N42 and *B. subtilis* MRHKN43 isolates and the fresh and dry weight of the plant is significantly enhanced by isolate *B. mycoides* N42 (Fig 4). Osman and Yin (2018) screened and isolated plant growth promoting bacteria associated with pea plant and found that most of the isolates were identified as *Bacillus* spp.

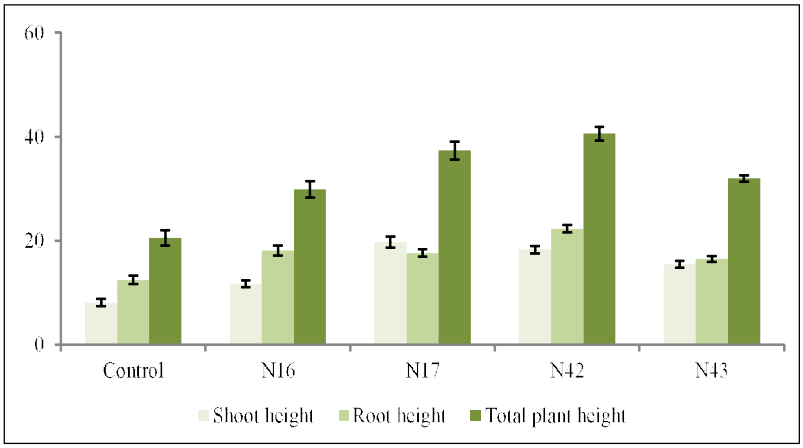

Fig 1: Black pepper cuttings treated with the *Bacillus* isolates (*B. thuringiensis* N16, *B. cereus* N17, *B. mycoides* N42 and *B. subtilis* N43) showing increased plant height compared to untreated (Control).

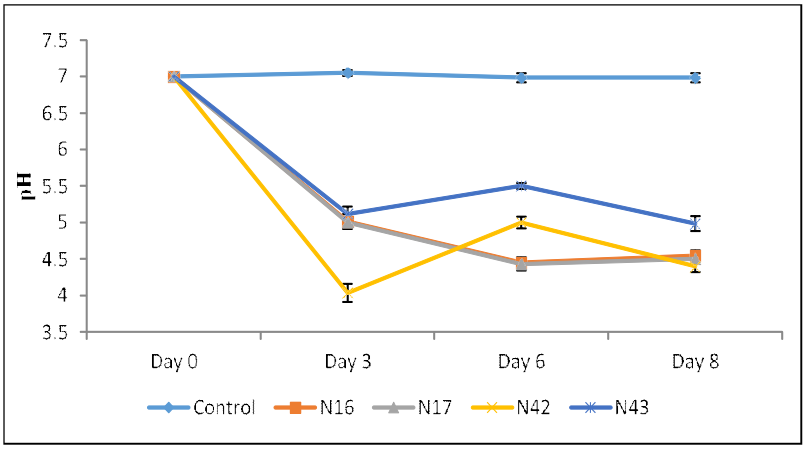

Fig 2: Phosphate solubilisation test: shows a decrease in pH in all the four isolates.

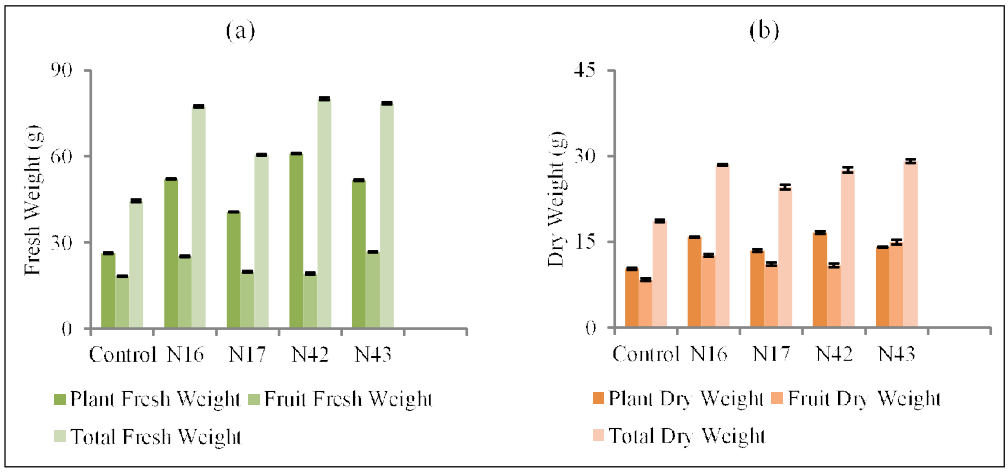

Fig 3: (a) and (b): Fresh and dry weight of French beans treated with the *Bacillus* isolates (*B. thuringiensis* N16, *B. cereus* N17, *B. mycoides* N42 and *B. subtilis* N43) showing increased plant growth compared to untreated (Control).

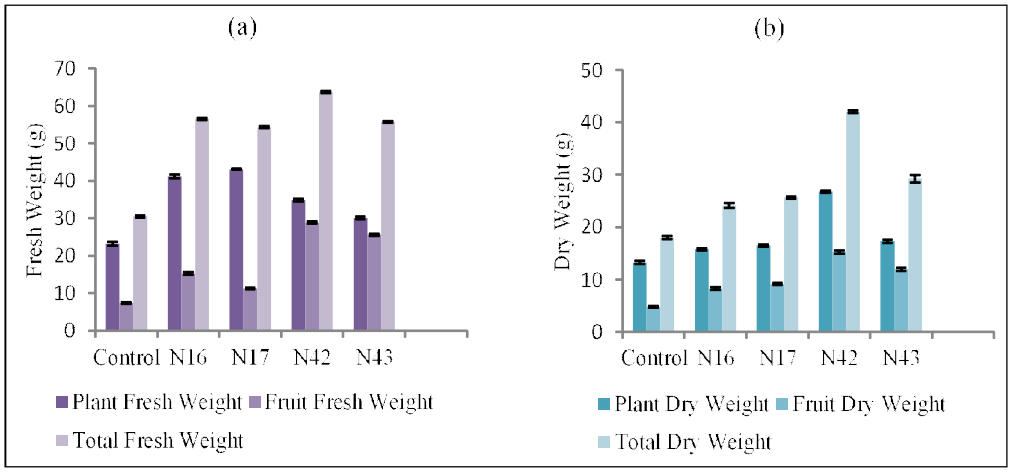

Fig 4: (a) and (b): Fresh and dry weight of Pea plant treated with the *Bacillus* isolates (*B. thuringiensis* N16, *B. cereus* N17, *B. mycoides* N42 and *B. subtilis* N43) showing increased plant growth compared to untreated (Control).

CONCLUSION

This study aims on isolating the PGPR associated with black pepper and screening for their plant growth promoting attributes and checking their ability to promote plant growth in greenhouse environment. As the selected isolates have the ability to promote plant growth on the host plants, test plants were also assayed to verify if the same isolates share similar capabilities. Thus, this study infer that all the four *Bacillus* spp. isolated from the rhizosphere of black pepper shows favourable results on both the bio-assays, *i.e.* they were able to significantly enhance the growth of the host plant (*Piper nigrum*) and the two test plants (*Phaseolus vulgaris* and *Pisum sativum*). Based on these findings, it is evident that these isolates can be employed to enhance the growth not only of black pepper but of the test plants as well; thus, offers a sustainable alternative as bio-fertilizers to improve plant growth of major important crop plants.

Conflict of interest: None.

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